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Transcription of the Rat and Mouse Proenkephalin Genes Is Initiated at Distinct Sites in Spermatogenic and Somatic Cells

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During spermatogenesis, several genes are expressed in a germ cell-specific manner. Previous studies have demonstrated that rat and mouse spermatogenic cells produce a 1,700-nucleotide proenkephalin RNA, while somatic cells that express the proenkephalin gene contain a 1,450-nucleotide transcript. Using cDNA cloning, RNA protection, and primer extension analyses, we showed that transcription of the rat and mouse spermatogenic-cell RNAs is initiated downstream from the proenkephalin somatic promoter in the first somatic intron (intron A_s). In both species, the germ cell cap site region consists of multiple start sites distributed over a length of approximately 30 base pairs. Within rat and mouse intron A_s, the region upstream of the germ cell cap sites is GC rich and lacks TATA sequences. A consensus binding site for the transcription factor SP1 was identified in intron A_s downstream of the proenkephalin germ cell cap site region. These features are characteristic of several previously described promoters that lack TATA sequences. Homologies were also identified between the proenkephalin and rat cytochrome *c* spermatogenic-cell promoters, including the absence of a TATA box, a multiple start site region, and several common sequences. This promoter motif thus may be shared with other genes expressed in male germ cells.

Spermatogenesis is a complex program of cellular differentiation that results in the formation of haploid spermatozoa. While this developmental sequence of events has been well characterized morphologically, a description of the molecular mechanisms regulating spermatogenic cell differentiation has only recently been initiated (18). Germ cell gene expression is highly stage specific, with different gene products being expressed at distinct phases of development. Both stage-specific transcriptional and translational regulation of early-transcribed mRNAs contribute to these differentiational changes (18, 19). Another characteristic of spermatogenic-cell gene expression is the presence of germ cell-specific transcripts not produced in somatic cells. These unique RNAs may be generated by multiple mechanisms, including transcription of genes selectively expressed in germ cells (5, 37, 49), utilization of distinct transcriptional initiation or termination sites (12, 39), and alternative RNA splicing.

The gene for the opioid precursor proenkephalin is expressed in both spermatogenic and somatic cells (13, 28–30). In the mouse and rat, spermatogenic cells produce a 1,700-nucleotide (nt) proenkephalin RNA, while somatic cells selectively express a 1,450-nt form. The proenkephalin gene is developmentally regulated during spermatogenesis, with the highest expression occurring in late pachytene spermatocytes and postmeiotic round spermatids (28, 29). The proenkephalin gene thus serves as a model for investigating germ cell-specific gene expression and its developmental regulation. Using cDNA sequencing, RNA protection, and primer extension analyses, we demonstrated that the germ cell forms of rat and mouse proenkephalin RNA are generated by alternative transcriptional initiation. The transcripts have multiple start sites distributed over a ~30-bp region within the first somatic intron of the rat and mouse proenkephalin genes.

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MATERIALS AND METHODS

Isolation of proenkephalin cDNA from mouse testis. A mouse testis cDNA library constructed in the *Eco*RI site of λgt10 (kindly provided by Ken Kleene, Biology Department, University of Massachusetts, Boston) was screened for proenkephalin-containing phage by hybridization to a *Pvu*II fragment from rat brain proenkephalin cDNA [pRPE-1(165-600) (21)]. The largest insert from the positive bacteriophage clones was isolated by *Eco*RI digestion and subcloned into the *Eco*RI site of pBluescript SK (Stratagene, San Diego, Calif.). Standard procedures were used throughout for the growth and isolation of bacteriophage and plasmids and the preparation of DNA fragments (34).

Isolation of rat and mouse proenkephalin genomic clones. A rat genomic library was constructed by G. Scherer by ligation of a *Sau*3A partial digest of rat liver DNA (15- to 20-kilobase [kb] fragments) into the *Bam*HI site of λ phage vector EMBL3. *E. coli* Q359 was infected with this library, and plaques were screened with the ^{32}P-labeled rat proenkephalin cDNA insert of pYSEC1 (51) by the method of Benton and Davis (3). Two positive clones were plaque purified and characterized by comparison of restriction maps with that reported previously by Rosen et al. (42). A 4.2-kb *Sal*I-*Eco*RI fragment containing 2.8 kb of upstream proenkephalin sequence, as well as somatic exons I and II, intron A (600 bases), and approximately 500 bases of intron B, was subcloned in the vector pIBI30 (International Biotechnologies, Inc.) to generate the plasmid pRESS2. This plasmid was used for the isolation of various fragments used in RNA protection experiments.

A mouse genomic library constructed by ligation of size-selected partial *Mbo*I-digested DNA into the *Bam*HI site of λJ1 was kindly provided by Yueh-hsiu Chien (Stanford University School of Medicine, Stanford, Calif.) (7). Bacteriophage (approximately 1.2×10^6) were screened with an *Sma*I fragment containing the 5′ end of the mouse testis proenkephalin cDNA (pMTP6). After isolation of positive

clones, phage DNAs (10 μg each) were digested with restriction enzymes, transferred to Gene Screen Plus (Dupont Co., Wilmington, Del.), and hybridized with the same *Sma*I probe to identify intron A-containing fragments. A *Pvu*II-*Sma*I fragment shown to contain mouse proenkephalin exon I, as well as portions of intron A and 81 bp of upstream sequence, was subcloned into the polylinker of pBluescript SK.

RNA and DNA probe preparation. Plasmids used for the synthesis of RNA probes were constructed in pBluescript SK by using appropriate rat or mouse proenkephalin genomic fragments. Radiolabeled antisense RNA (>10^9 cpm/μg) probes were generated from linearized plasmids by using [α-^{32}P]UTP (800 Ci/mmol) (Amersham Corp., Arlington, Heights, Ill.) and either T7 or T3 RNA polymerase (36). DNA probes used in the analysis of RNA and DNA gel blots were labeled by random priming (27).

RNA protection analyses. S1 nuclease protection analysis was performed essentially as described by Blum et al. (4). Radiolabeled transcripts (5 × 10^4 cpm) were hybridized to either total or poly(A)-selected RNA (5 to 25 μg) in a final volume of 30 μl of buffer containing 40% formamide, 600 mM NaCl, 4 mM EDTA, and 40 mM Tris hydrochloride (pH 7.4) at 68°C for 12 to 16 h. Three hundred microliters of S1 nuclease buffer (300 mM NaCl, 30 mM sodium acetate [pH 4.8], 3 mM $ZnCl_2$, 20 μg of sonicated salmon sperm DNA) and 2,000 U of S1 nuclease (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) were then added, and samples were incubated at 56°C for 1 h. After incubation, each sample received 5 M ammonium acetate (4 μl), 0.5 M EDTA (1 μl), and 1 μl of yeast RNA (10 mg/ml) and was extracted with an equal volume of phenol-chloroform. Samples were then precipitated with an equal volume of isopropanol, dried, and suspended in loading buffer containing 97% formamide. The protection fragments were then heat denatured at 85°C for 5 min and separated on a 6% polyacrylamide gel containing 8 M urea.

For RNase protection, conditions for hybridization were identical to those used for S1 nuclease analysis. After hybridization, 300 μl of digestion buffer containing 10 mM Tris hydrochloride (pH 7.5), 300 mM NaCl, 5 mM EDTA, 40 μg of ribonuclease A per ml, and 2 μg of RNase T1 per ml were added, and samples were incubated at 30°C for 45 min. Samples then received 10 μl of 20% sodium dodecyl sulfate and 2.5 μl of proteinase K (20 mg/ml) and were incubated for an additional 15 min at 37°C. They were then extracted, precipitated, and analyzed on polyacrylamide gels as described above.

In certain instances, RNA fragments of known length and sequence were generated by RNase protection for use as standards to take into account small differences in mobility between protected RNA fragments and DNA sequencing ladders used to estimate their sizes.

Primer extension. Continuously labeled primers (22) were generated from denatured, closed circular plasmid DNA (2 to 3 μg) by using either T7 or T3 primers (75 ng). Reaction conditions were as follows: 40 mM Tris hydrochloride (pH 7.5); 1 mM each dATP, dTTP, and dGTP; 10 mM dithiothreitol; 100 μCi of [α-^{32}P]dCTP (800 Ci/mmol); and 15 U of Klenow fragment of DNA polymerase I (Pharmacia Inc., Piscataway, N.J.). After incubation at 37°C for 60 min, the reaction mixture was digested with *Nar*I. Digests were phenol-chloroform extracted and ethanol precipitated, and the labeled primers were then purified on 6% polyacrylamide gels containing 8 M urea.

Labeled primer (5 × 10^3 to 10 × 10^3 cpm) was hybridized to 5 μg of poly(A) RNA in 10 μl of aqueous hybridization buffer (0.2 M KCl, 0.2 M Tris [pH 8.3], 10 mM EDTA) at 70°C for 12 to 16 h. Ninety microliters of a reverse transcriptase mix was then added, and the reactions were incubated at 37°C for 30 min. The reverse transcriptase mix consisted of 50 mM Tris (pH 8.3), 3 mM dithiothreitol, 7 mM $MgCl_2$, 0.9 mM each dATP, dCTP, dTTP, and dGTP, 360 U of RNAsin per ml, (Promega Biotec, Madison, Wis.), and 50 mM KCl. The extension reactions were then phenol-chloroform extracted, ethanol precipitated, and separated on 6% polyacrylamide–8 M urea sequencing gels.

DNA sequencing. The complete nucleotide sequences of genomic and complementary DNAs were determined for both strands by the dideoxy-chain termination method (43), using appropriate restriction fragments subcloned into pBluescript KS. Synthetic oligonucleotide sequencing primers based on specific insert sequences were also used when necessary. Sequence data were analyzed with the Staden and Genetics Computer Group software packages (11, 46) on a VAX/VMS 11/750 computer.

RNA preparation and gel blot analysis. Tissues for RNA preparations were isolated from adult male rats and mice (CD-1 in both cases) and stored at −80°C. Mouse spermatogenic cells were purified from freshly isolated testes to greater than 90% homogeneity by unit gravity sedimentation as described previously (29). Methods for the preparation of total and poly(A)-selected RNA, formaldehyde gel electrophoresis, membrane transfer, and hybridization have been described previously (27, 38). RNAs were run on either 1.0 or 1.5% agarose gels.

RESULTS

Sequence of proenkephalin cDNA from mouse testis. Screening of the mouse testis cDNA library (approximately 50,000 phage) yielded five positive phage, three of which contained the largest insert size (approximately 1.5 kb). The cDNA sequence deduced from one of these 1.5-kb inserts is shown in Fig. 1. It is 1,408 base pairs (bp) in length, excluding the poly(A) tail, and contains a long open reading frame (ORF) of 269 amino acids beginning at nucleotide 1. This coding region is highly homologous to that previously reported for proenkephalin from rat brain and testis (96% identity at the protein level) (21, 50, 52). Similar to rat, bovine, and human proenkephalin (42), the mouse form contains seven enkephalin or enkephalin-related sequences that are bracketed by pairs of basic amino acid residues. One interesting divergence found in mouse proenkephalin is a glycine-to-serine conversion (nucleotide 580) that yields a unique enkephalin-containing octapeptide, Met-enkephalin-Arg-Ser-Leu (Fig. 1). Our amino acid sequence for mouse proenkephalin generally agrees with that reported by Zurawski et al. (53), with one exception. The T-cell cDNA sequence lacks the serine codon (AGC) present at nucleotide 560 to 562 of the testis cDNA.

The nucleotide sequence of the 305-nt 3′-untranslated region of the mouse testis RNA also shares considerable sequence homology with the corresponding region of rat somatic proenkephalin mRNA (86% identity) (21, 52). However, the sequences upstream of the proenkephalin ORF in the mouse testis RNA (from nucleotide −4 to −296) were not homologous to the 5′-untranslated region of the rat somatic transcript (exon I_s) (52). The location of this sequence divergence corresponds precisely to the splice site for rat somatic exons I_s and II_s (42). In fact, the 5′ end of the mouse testis mRNA was very similar to the partially se-

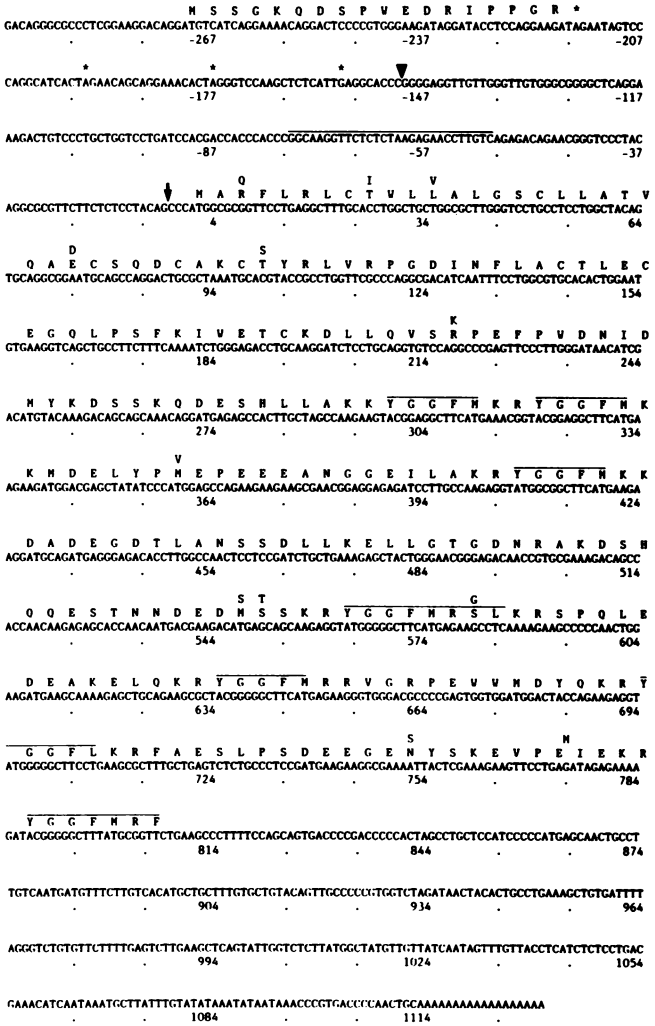

FIG. 1. Nucleotide sequence of the mouse spermatogenic-specific form of proenkephalin RNA. The sequence data were obtained from the cDNA-containing plasmid pMTP6. The two ORFs are shown in single-letter code above the nucleotide sequence. Intervening stop codons in frame with the first ORF are indicated by asterisks. Differences between the mouse and rat (21, 41, 50, 52) proenkephalin protein sequences are shown above the appropriate amino acids. The mouse and rat proenkephalin sequences differ at amino acid 12 on the basis of the reported sequences for the rat gene (42) and rat testis cDNA (50), but are identical on the basis of the sequence for the rat brain cDNA (21, 52). Overlining indicates the locations of (i) enkephalin and enkephalin-containing peptides bracketed by pairs of basic amino acids and (ii) the 29-bp region upstream of the proenkephalin ORF capable of forming a stem-loop structure. The arrow indicates the site corresponding to the exon I-exon II splice junction for the rat somatic (brain) proenkephalin mRNA. The *Sma*I site used to generate a cDNA probe specific to the mouse intron A_s region is also indicated (▼).

quenced 3′ end of the intron between exons I_s and II_s of the rat proenkephalin gene (intron A_s) (42). Similar results were recently reported for the germ cell-specific proenkephalin RNA from the rat (50). Subsequent sequencing of intron A_s from the mouse and rat proenkephalin genes established that the 5′-terminal sequences of the cloned testis cDNAs for both species are derived entirely from this region (see below and reference 15).

The mouse testis transcript contains an additional methionine-initiated ORF upstream of the proenkephalin reading frame beginning at nucleotide −271 that encodes an 18-amino-acid peptide (Fig. 1). No significant sequence homology was found between this amino acid sequence and that of other proteins in the National Biomedical Research Foundation protein data base. A total of four in-frame stop codons are present downstream of this smaller ORF. The 5′ region also contains a near-perfect inverted repeat located between positions −76 and −48 that is capable of forming a stable hairpin structure (12 of 13 complementary pairs separated by three nucleotides; Fig. 1).

Protection analysis of rat and mouse germ cell transcripts. The rat genome contains a single proenkephalin gene (42), and Southern analysis of genomic DNA indicated that the same was true for the mouse (see below). On the basis of the sequences of their respective cDNAs, the germ cell-specific forms of proenkephalin mRNA in both the rat and mouse may be the result of transcription from a unique initiation site(s) present within intron A_s. Alternatively, it was possible that the cDNAs were incomplete at their 5′ ends (e.g., missing exon I_s sequences), and the germ cell transcripts were generated by cell-specific splicing (or a combination of alternative initiation and splicing). RNase and S1 nuclease protection analyses were performed to distinguish between these possible mechanisms.

For protection studies in the rat, a 733-bp *Sac*I-*Pst*I fragment containing the region of interest (intron A_s as well as the 3′ and 5′ ends of exons I_s and II_s, respectively) was subcloned into pBluescript KS for generation of antisense RNA probes (Fig. 2A). Protection experiments were carried out on RNA from rat testis and rat brain to compare the exon pattern for the 1,700-nt, germ-cell transcript with that for the smaller 1,450-nt form. Rat kidney RNA was used as a negative control.

Two groups of protected fragments were observed in rat brain having sizes of 101 to 103 and 80 to 82 nucleotides, respectively (Fig. 2B). These sizes agree well with those predicted for the somatic exon I and II fragments present within the *Sac*I-*Pst*I probe (101 and 82 nt, respectively). No bands were detected in the rat kidney sample. As predicted from the rat testis cDNA sequence (50), S1 nuclease and RNase analyses of rat testis RNA yielded a much larger protected fragment (>300 nt; Fig. 2B). This presumably represented the region extending in the 5′ direction from the *Pst*I site in somatic exon II to an unknown location within intron A_s. Additional testis bands that comigrated with the exon I_s and II_s protection fragments seen in rat brain were evident, but only when larger amounts of total RNA [or poly(A) RNA] were used (Fig. 2B). The relative intensities of these bands were identical to those seen for the 1,450-nt somatic transcript. Rat and mouse testis are known to contain the latter mRNA, although at much lower concentrations than the larger germ cell form (28). In addition, the abundances of total proenkephalin transcripts in rat brain and testis are similar (see Fig. 4). The 101- and 82-nt protection fragments observed in rat testis thus were probably not derived from the 1,700-nt proenkephalin RNA, but instead represented the less-abundant, 1,450-nt transcript in rat testis.

The *Sac*I-*Pst*I rat probe initially used in these studies did not contain the 5′ end of somatic proenkephalin exon I (Fig. 2A). A *Pvu*II-*Sac*I probe containing 52 nt of this exon as well as 81 nt of upstream region was used to determine the presence of these sequences in rat testis. After short-term autoradiographic exposure, two major bands corresponding

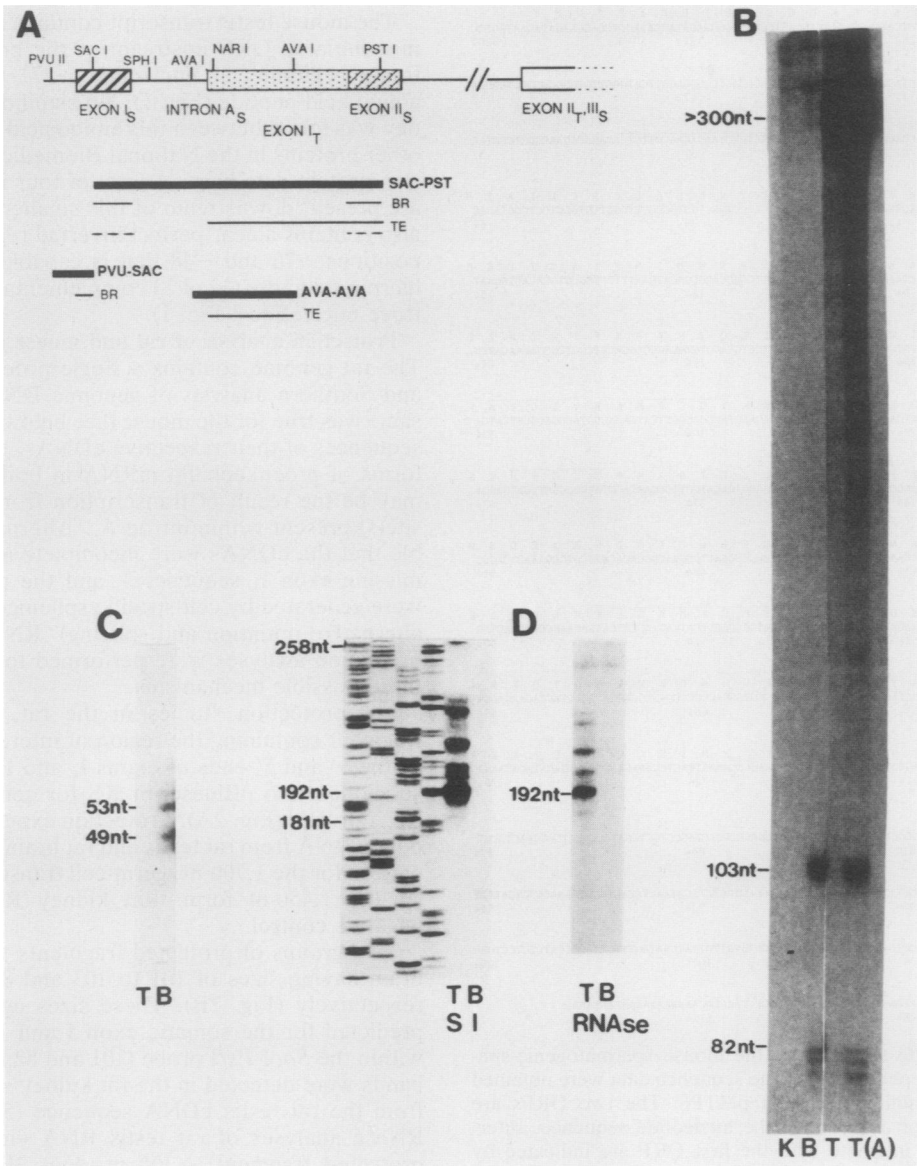

FIG. 2. Protection analysis of rat proenkephalin RNA. (A) Schematic showing the exon-intron structure for the 1,450-nt, somatic form of rat proenkephalin mRNA (exons I_s, II_s, III_s and intron A_s) and the positions of restriction sites used in generating various probes. RNA probes used in protection analysis (▬) and the relative positions of protected products observed in rat testis (TE) and brain (BR) (—— and – – –) are given below the schematic (dashed lines indicate minor protection fragments). The exon positions for the 1,700-nt, spermatogenic-cell form of rat proenkephalin RNA determined by protection studies are also shown (exon I_t [▨] and exon II_t). (B, C, and D) Autoradiograms showing the results of RNA protection experiments using the *Sac*I-*Pst*I, *Pvu*II-*Sac*I, and *Ava*I-*Ava*I RNA probes, respectively. S1 nuclease was used exclusively in the experiments shown except in the case of panel D, in which RNase protection results are also given. Total RNA (30 μg) from rat kidney (K), brain (B), and testis (T) or rat testis poly(A) RNA [T(A); 15 μg] was analyzed. DNA-sequencing ladders were used to estimate the sizes of the various protected products. The DNA-sequencing ladder shown in panel D was generated by using pSV2CAT. A *Nar*I-*Pst*I probe (258 nt) and its protection fragment generated with rat testis total RNA (181 nt) were used in the latter experiments as additional size standards.

to exon I (49 and 53 nt) were observed in rat brain (Fig. 2C). The presence of two bands may reflect heterogeneity in the location of the proenkephalin cap site or may be an artifact of the S1 nuclease protection method. Similar bands were not detectable in the rat testis sample after an equivalent exposure time (Fig. 2C).

The exact length of somatic intron A sequence contained within the rat testis-specific transcript was determined by using an *Ava*I-*Ava*I probe (Fig. 2A). No signal was observed in rat brain, consistent with the absence of intron A_s sequences in the 1,450-nt transcript (Fig. 2D). A major band of approximately 192 nt and minor species of 198 to 201 nt, 211 nt, and 226 nt were detected in rat testis. This broad band pattern was obtained whether S1 nuclease or RNase digestion was used (Fig. 2D). It therefore seemed likely that these results represented heterogeneity in the germ cell transcripts. On the basis of protection and cDNA sequencing data, the rat testis-specific proenkephalin RNA is composed of at least two exons: one consisting of various lengths (340 to 374 nt) of somatic intron A together with the 141-nt exon

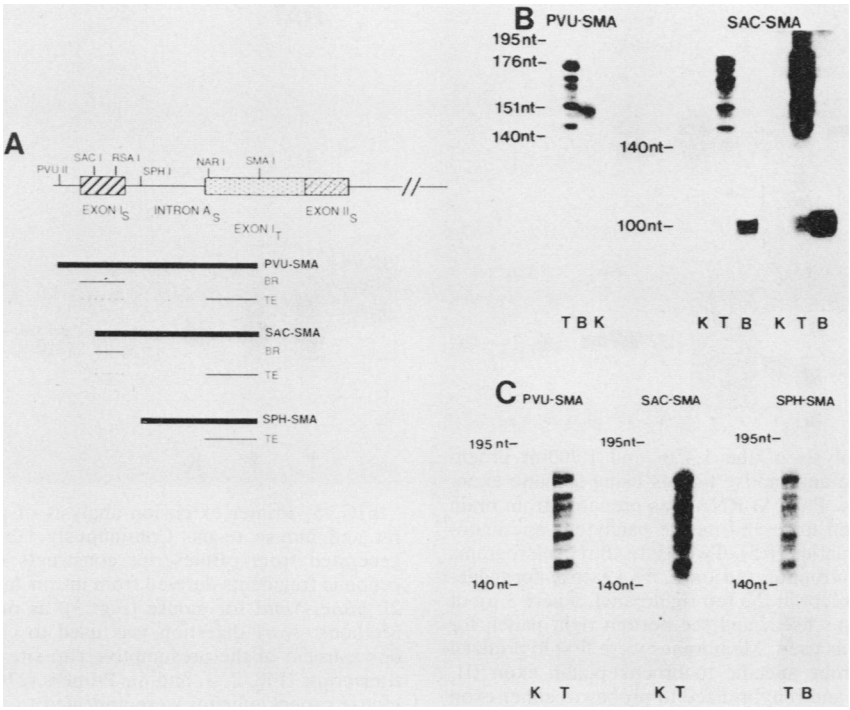

FIG. 3. RNase and S1 nuclease protection analyses of the 1,450-nt and 1,700-nt proenkephalin RNAs in mouse brain and testis. (A) The exon-intron relationships for the two mouse proenkephalin transcripts are given on the basis of comparisons of the mouse testis cDNA and genomic sequences with the rat genomic sequence together with the results of protection experiments. The locations of relevant restriction sites and RNA probes and their resultant protection products for mouse brain (BR) and testis (TE) are indicated as in Fig. 2. (B) S1 nuclease protection analysis of mouse kidney (K), brain (B), and testis (T) poly(A) RNAs using the *Pvu*II-*Sma*I and *Sac*I-*Sma*I RNA probes. Two autoradiographic time exposures of the *Sac*I-*Sma*I gel (16 and 110 h) are shown. The positions of major protected fragments and of a *Nar*I-*Sma*I RNA probe (195 nt) and its testicular protection product (140 nt) are indicated. (C) Results of RNase protection analysis of mouse brain and testis with each of the three RNA probes shown in panel A. Ten micrograms of poly(A) RNA was used in each protection experiment.

II_s, and the other being equivalent to exon III_s (978 nt) (Fig. 2A).

Protection analysis in the mouse was performed with genomic clones containing exon I_s and intron A_s of the mouse proenkephalin gene. These were isolated by using a *Sma*I fragment containing the 5′ end of the mouse testis cDNA as a probe (Fig. 1). Restriction fragments that hybridized to this probe were identical among the three clones and were the same as those observed with restriction-digested mouse genomic DNA (not shown). The mouse genome thus appears to contain a single copy of the proenkephalin gene. A *Pvu*II-*Sma*I genomic fragment was subsequently subcloned and shown to contain sequences homologous to that portion of the rat proenkephalin gene extending from 81 bp upstream of the somatic cap site through exon I_s and into intron A_s (Fig. 3A).

Protection experiments were performed on RNA from mouse brain, testis, and kidney. Three antisense probes were used that extended different lengths upstream of the *Sma*I site within the mouse intron A_s region (to *Pvu*II, *Sac*I, and *Sph*I sites; Fig. 3A). No protection was seen with mouse kidney, while mouse brain protected a major band of approximately 150 nt with the *Pvu*II-*Sma*I probe and a 100-nt band with the *Sac*I-*Sma*I probe (Fig. 3B). The sizes of these bands agree with those predicted for mouse exon I_s on the basis of comparison of the mouse and rat proenkephalin genomic sequences (data not shown). As expected, no bands were observed in mouse brain with the *Sph*I-*Sma*I probe, which contains only intron A_s sequences. The band pattern obtained with mouse testis RNA was the same for all three probes: major bands of 143, 151, and 176 nt and minor species of approximately 165 and 170 nt (Fig. 3C). These protection fragments thus represent sequences present within the 300-bp region of intron A_s defined by the *Sma*I-*Sph*I probe. The observed size heterogeneity was apparently not due to a technical artifact, since it was independent of the digestion protocol used (Fig. 3B and C). On the basis of these data, the major mouse germ cell transcripts contain 290, 298, and 327 nt, respectively, of the 3′ end of mouse intron A_s.

RNA gel blot analysis. As for rat testis, low but detectable amounts of exon I_s bands were observed in mouse testis after prolonged autoradiography (see data for *Sac*I-*Sma*I probe [Fig. 3B]) that apparently represented the much less-abundant 1,450-nt proenkephalin transcript. Therefore, the 1,700-nt, germ cell form of proenkephalin RNA from neither species appeared to contain exon I_s sequences. This conclusion was confirmed by RNA gel blot analysis of brain and testis poly(A) RNA with DNA probes to either exon I_s or the 3′ region of intron A_s of the appropriate species. With the intron A_s probes, hybridization was seen only to the 1,700-nt, germ cell forms in rat and mouse testis (Fig. 4). Conversely, the exon I_s probes hybridized to the 1,450-nt transcripts in brain but not to the larger germ cell transcripts in testis. Thus, formation of the 1,700-nt proenkephalin RNA in rat and mouse spermatogenic cells involves transcriptional initiation from a unique region within the proenkephalin gene. In agreement with the results from protection

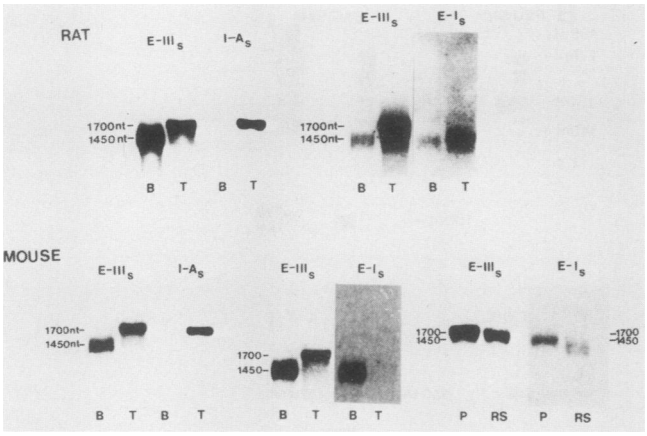

FIG. 4. RNA blot analysis of the 1,450- and 1,700-nt proenkephalin transcripts in rat and mouse tissues using somatic exon- and intron-specific probes. Poly(A) RNA was prepared from brain and testis of adult rats and mice and mouse pachytene spermatocytes (P) and round spermatids (RS). Twenty to thirty micrograms of poly(A) RNA was electrophoresed on 1.5% agarose–formaldehyde gels in each case, except in the top right panel, where 3 μg of rat brain poly(A) RNA was used, and the bottom right panel, for which a 1% agarose gel was used. Membranes were first hybridized to a ^{32}P-labeled DNA probe specific to proenkephalin exon III$_s$ (E-III$_s$) and then stripped and rehybridized to probes to either exon I$_s$ (E-I$_s$) or the 3′ end of intron A$_s$ (I-A$_s$) of the appropriate species. For the rat, the exon I$_s$ and intron A$_s$ probes consisted of *Pvu*II-*Sph*I and *Ava*I-*Ava*I fragments derived from pRESS2, respectively (Fig. 2A). The mouse exon I$_s$ probe was a *Pvu*II-*Rsa*I genomic fragment (Fig. 3A), and the *Sma*I fragment derived from the 5′ end of the mouse testis cDNA, pMTP6, was used as the mouse intron A$_s$ probe (see Fig. 1). The exon III$_s$ probe was derived from rat brain proenkephalin cDNA [pRPE-1(165–600)] (21).

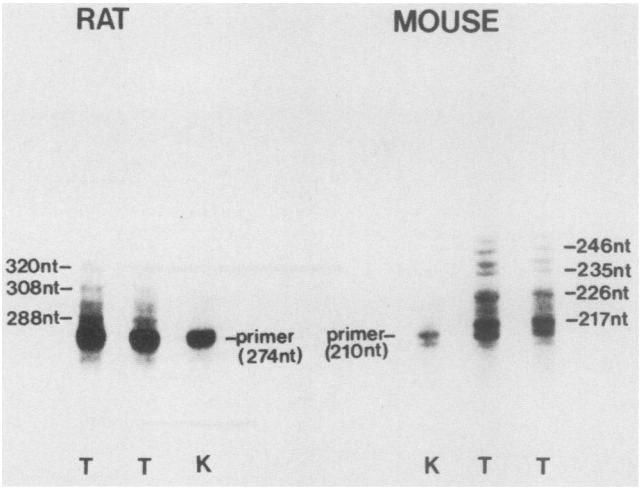

FIG. 5. Primer extension analysis of proenkephalin RNA from rat and mouse testis. Continuously labeled DNA primers were generated from pBluescript constructs containing proenkephalin genomic fragments derived from intron A$_s$ (p*Ava*I-*Ava*I for rat [Fig. 2]; p*Sac*I-*Sma*I for mouse [Fig. 3]) as described in Materials and Methods. *Nar*I digestion was used to create primer 3′ ends just downstream of the presumptive cap site regions for the germ cell transcripts (Fig. 2, 3, and 6). Primers (274 nt for rat and 210 nt for mouse proenkephalin) were annealed to poly(A) RNA (5 μg) from rat or mouse testis and mouse kidney. After extension with reverse transcriptase, products were separated from unextended primer on denaturing polyacrylamide gels. Sequencing ladders were generated as size standards by using the same template-primer combinations used in the synthesis of the continuously labeled primers.

analysis, small amounts of the 1,450-nt proenkephalin mRNA were also detected in rat and mouse testis with the exon I$_s$ probe (Fig. 4). Interestingly, RNA blots also revealed the presence of low amounts of the 1,450-nt proenkephalin transcript in purified mouse pachytene spermatocytes and round spermatids, in addition to the predominant 1,700-nt RNA (Fig. 4). Mouse spermatogenic cells thus may be capable of initiating transcription from the somatic cell promoter of the proenkephalin gene, at least to a small extent.

Primer extension analysis. The results from RNA protection studies demonstrated that the somatic and germ cell forms of proenkephalin RNA are transcribed from distinct initiation sites. The cap sites for the rat and mouse germ cell transcripts appeared to be located within intron A$_s$, since the 5′ ends of their cDNAs terminate in this region. However, it was possible that a small, additional germ cell exon existed upstream of the region examined by RNA protection (81 bp upstream of the somatic proenkephalin cap site). In fact, potential 3′-splice acceptor sequences (6) exist in the vicinity of the putative rat and mouse germ cell cap sites (see Fig. 6). Primer extension was therefore performed to determine the 5′ end of the 1,700-nt transcripts.

Initial experiments using short oligonucleotide primers (20 to 30 nt) proved unsuccessful. It seemed likely that this was due to secondary structure associated with the 5′ end of the rat and mouse germ-cell transcripts (Fig. 1; see below) that could interfere with primer annealing and/or access of enzyme to the hybrid. Longer primers that would anneal to RNA under conditions sufficiently stringent to prevent the formation of hairpin structures were therefore used. Antisense DNA primers for rat (274-nt) and mouse (210-nt) germ cell transcripts were generated from *Ava*I-*Ava*I and *Sac*I-*Sma*I constructs, respectively (Fig. 2A and 4A). *Nar*I, which cleaves within intron A$_s$ of both species just downstream of the putative germ cell cap site regions, was used to form the 3′ end of each primer. Multiple specific extension products were obtained with these primers and mouse or rat testicular poly(A) RNA (Fig. 5). These extensions ranged in length from 7 to 36 nt for mouse testis and from 14 to 46 nt in the rat. Extension products were not observed with mouse kidney poly(A) RNA (Fig. 5). For both species, the testis band patterns were essentially identical to those obtained from RNA protection analysis (Fig. 2 and 4), and the 5′ ends of the germ cell transcripts predicted by the two methods are in very close agreement (Fig. 6). Transcriptional initiation of the family of ~1,700-nt proenkephalin transcripts in rat and mouse spermatogenic cells therefore occurs over a 30- to 33-bp region within intron A$_s$.

Sequence analysis of intron A$_s$ regions for the rat and mouse proenkephalin genes. It was of interest to compare the sequences surrounding the transcriptional initiation sites for the rat and mouse germ cell-specific transcripts. Intron A$_s$ for both proenkephalin genes was therefore completely sequenced (Fig. 6). The rat intron is 63 bp longer than that of the mouse (549 versus 486 bp), the larger size being essentially accounted for by two additional sequences of 19 and 43 bp. Exclusive of these extra sequences, the two introns are highly homologous (87% identity). The sequence for rat intron A$_s$ presented here contains a 38-bp region not found in the rat intron sequence recently reported by Garrett et al. (15) (Fig. 6). This discrepancy is apparently not due to sequence variation between different animal strains, since in both studies the rat proenkephalin genomic clones used for

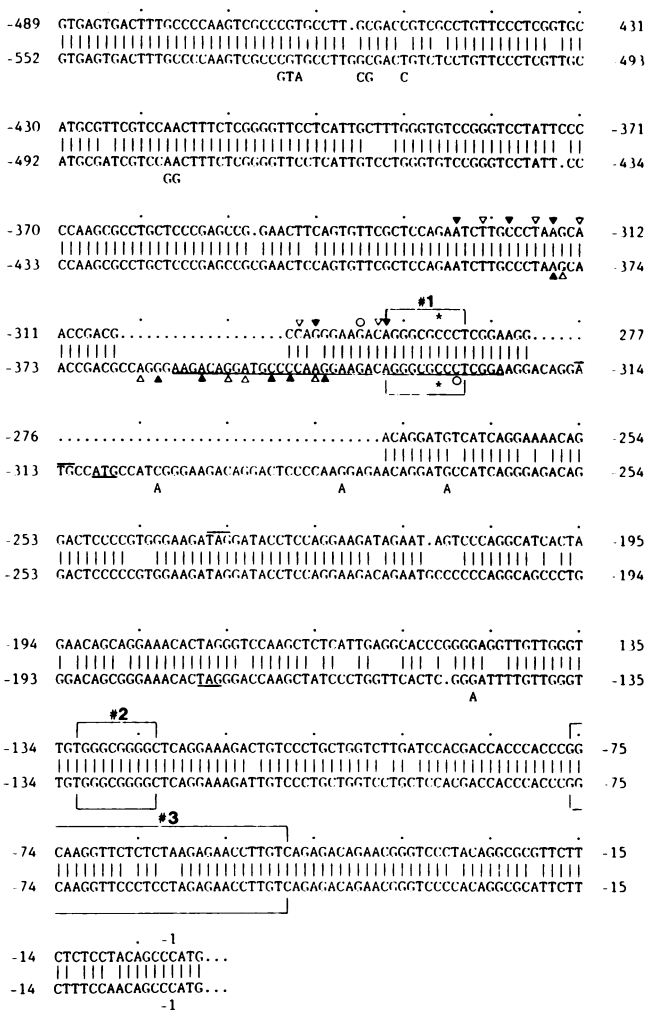

FIG. 6. Nucleotide sequence comparison of intron A_s for mouse and rat proenkephalin. The mouse sequence is a composite of genomic and cDNA sequence data. Gaps have been inserted to maximize the homology between the two introns. The sense-strand sequences are presented, and nucleotides are numbered relative to the first nucleotide in the proenkephalin translational start codon. Transcriptional initiation sites as determined by RNA protection (▽) and primer extension (▼) analyses are shown. The 5′ ends of the rat (50) and mouse testicular cDNAs are also indicated (○); asterisks indicate the *Nar*I cleavage sites used to generate primers for extension analysis. The 38-bp region not present in the rat intron A_s sequence reported by Garrett et al. (15) is underlined, and individual nucleotide differences with the latter sequence are shown below the rat sequence. Codons defining the start and termination of the first and second ORFs upstream of the proenkephalin reading frame in the rat germ cell transcript are indicated by over- and underlining, respectively. Structures discussed in the text are indicated by numbered brackets: #1, the 10-bp palindrome containing a GC box; #2, the SP1 consensus binding sequence; #3, a contiguous inverted repeat capable of forming a hairpin structure.

sequencing were isolated from the same library (15, 42). The 3′-terminal sequence of rat intron A_s reported here is identical to the 5′ end of the 1,700-nt rat germ cell transcript (50), including the first six nucleotides which are contained within the 38-bp region not found by Garrett et al. (Fig. 6).

Both introns contain regions that are repetitive, including a 10-bp perfect palindrome and a 28-bp near-perfect palindrome (positions −294 and −74, respectively, in the mouse, and −336 and −74 in the rat [relative to the translational start site for the proenkephalin reading frame]) (Fig. 6; structures #1 and 3). The larger inverted repeat corresponds to the previously noted hairpin structure present in the mouse 1,700-nt transcript. In the rat transcript, this hairpin contains one less complementarity (11 of 13 paired bases). Sequences corresponding to TATA and CAAT transcriptional elements are not present upstream of the transcriptional initiation region within either rat or mouse intron A_s. The sequences upstream of the cap site region are GC rich (approximately 60% for both introns), and a recognition sequence for the RNA polymerase II transcriptional activator SP1 (TGGGCGGGGC) (25) is present downstream of the putative transcription start region at nucleotide −130 in the mouse and −131 in the rat (Fig. 6; structure #2). The 10-bp palindrome also contains a GC box (GGGCGCCC). Consensus binding sites for the transcriptional factors AP1, AP3, AP4, AP5, CREB, NF1, and octamer-binding proteins (24) were not found in either intron A_s region.

Similarly, the promoter for the spermatogenesis-specific cytochrome c_t gene has no TATA-like structure, contains multiple transcription start sites, and is GC rich (60%) (47). Comparison of the proenkephalin and cytochrome c_t germ cell promoters revealed multiple common sequences ranging in length from 7 to 13 bp (Fig. 7). These structures included the previously mentioned 10-bp, GC box-containing palindrome in which the sequence is reversed in the cytochrome c_t promoter. Of particular interest is the similar positioning of many of these common sequences relative to the transcriptional initiation regions of each gene. Additional homologies with the cytochrome c_t gene were identified upstream of the proenkephalin intron A_s region (not shown). Another potentially significant feature is the presence of the sequence CAG in the vicinity of several of the germ cell cap sites identified within the three genes (Fig. 6 and reference 47).

Comparison of mouse and rat proenkephalin sequences with other genes expressed in spermatogenic cells (5, 10, 16, 20, 23, 35, 47) identified several homologies (data not shown). The functional significance of these sequences in spermatogenesis-specific gene transcription remains to be determined. One of these, the sequence GGGTGGG (positions −77 in the mouse gene and −77 and −968 in the rat), was recently shown to occur twice within the germ cell promoter for the human *PGK2* gene as functionally defined in transgenic mice (41). It should be noted, however, that this sequence is also present in the 5′-flanking region of the somatic *PGK1* gene (1).

DISCUSSION

The mechanisms regulating gene expression in spermatogenic cells appear to differ markedly from those operating in somatic cells at several levels. In the case of the mouse and rat proenkephalin genes, germ cell-specific transcripts are generated by initiation from a region distinct from that used by somatic cells. Using RNase protection analysis, Garrett et al. concluded that rat germ cell proenkephalin RNA was formed by alternative splicing (15). This conclusion was based on the assumption that testicular RNase protection fragments representing proenkephalin exon I_s were derived from the 1,700-nt transcripts. However, RNA blot analysis has now demonstrated that exon I_s sequences detected by protection analysis in rat and mouse testis are derived from the much-less-abundant 1,450-nt form of proenkephalin mRNA. The inability to discern the 1,450-nt transcript on RNA blots of adult testis in previous studies (13, 28–30) was

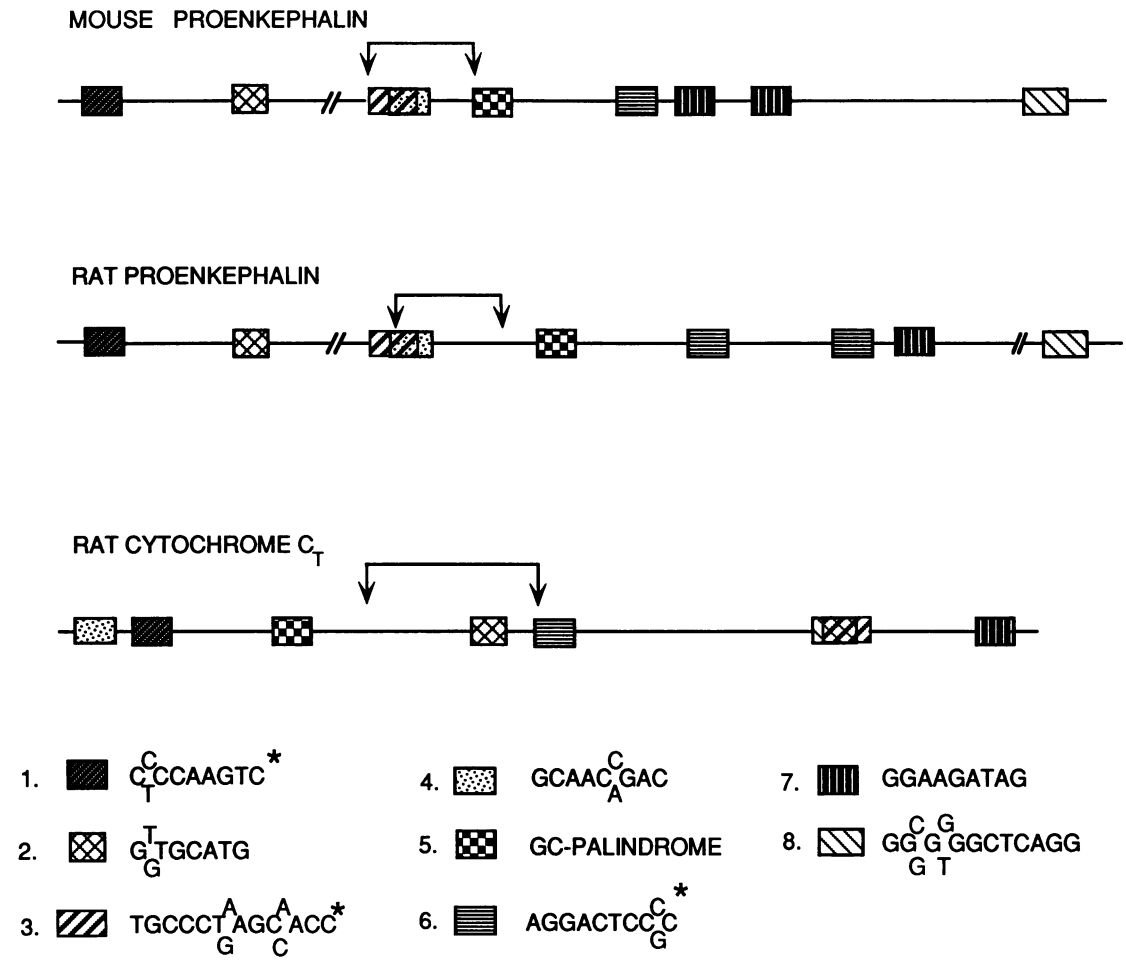

FIG. 7. Distribution of common sequences found in the proenkephalin and rat cytochrome c_t spermatogenesis-specific promoters. Only those sequences found in mouse and rat intron A_s are shown. The sequence of the GC palindrome is AGGGCGCCCT in proenkephalin and TCCCGCGGGA in cytochrome c_t. Sequences marked with an asterisk are present as the reverse complement in the cytochrome c_t gene. Brackets mark the transcriptional initiation regions for each germ cell promoter. The locations of homologous sequences in the rat (r) and mouse (m) proenkephalin genes were as follows (refer to Fig. 6): 1, −502 (m) and −565 (r); 2, −435 (m) and −497 (r); 3, −322 (m) and −384 (r); 4, −314 (m) and −376 (r); 5, −293 (m) and −336 (r); 6, −255 (m) and −293 and −255 (r); 7, −242 and −223 (m) and −242 (r); 8, −130 (m and r).

apparently due to the use of probes that recognized both forms of proenkephalin RNA together with the much greater abundance of the 1,700-nt transcripts.

The rat and mouse germ cell-specific RNAs contain proenkephalin reading frames identical to that present in the rat somatic transcript and relatively large 5′-untranslated regions characterized by the presence of one or more short ORFs. The contexts of the translational initiation codons for these upstream ORFs are at least as translationally favorable as that for the proenkephalin reading frame, on the basis of comparisons with the consensus sequence for translational initiation codons (32). Upstream ORFs can exert strong inhibitory effects on translation from downstream initiation sites (31), and it is likely that the relatively inefficient translation of the 1,700-nt proenkephalin RNAs in mouse and rat germ cells (15, 26) is due at least in part to the presence of upstream ORFs. The potential for these transcripts to form hairpin structures within 50 nucleotides of the proenkephalin start codon may also decrease translation from this site (33).

Little is known regarding the nature of *cis*-acting regulatory elements mediating spermatogenic-cell-specific gene transcription. Proenkephalin expression in rat and mouse testis offers the opportunity to compare the promoter preferences of spermatogenic cells and somatic cells within the same gene. The somatic and germ cell-specific transcriptional initiation regions in the proenkephalin gene are located within approximately 320 bp of each other, and it is therefore possible that they share common promoter elements. However, the proenkephalin germ cell promoter presumably consists at least in part of distinct elements. The upstream somatic promoter contains canonical CAAT and TATA boxes (42), while the germ cell cap site region does not. The absence of TATA sequences likely explains the much broader pattern of transcriptional initiation of the germ cell RNAs, since such sequences are involved in determining the location of RNA polymerase II initiation and their removal can lead to multiple start sites (2). Other genes lacking TATA sequences have been described, such as those coding for human X-linked phosphoglycerate kinase (44), mouse cyclic AMP-dependent protein kinase (9), human porphobilinogen (8), and hamster hydroxymethylglutaryl coenzyme A

reductase (40). The promoters for these genes contain multiple transcriptional start sites as well as GC-rich regions and SP1-binding sites, properties also associated with the germ cell promoters for the rat and mouse proenkephalin genes. The rat cytochrome c_t gene expressed during spermatogenesis also possesses similar characteristics (47). This promoter motif therefore may be utilized by both spermatogenic and somatic cells. The SP1-binding site in the proenkephalin germ cell promoter is located downstream of the presumptive cap site region, as is the case for the somatic rat cytochrome c_s and interleukin-1 alpha genes (14, 17). This SP1 site also may influence transcription at the more-upstream, somatic promoter of the proenkephalin gene. The possible role of the SP1 recognition sequence, as well as other potential promoter features identified here, in germ cell transcription of the proenkephalin gene merits further investigation. Functional analysis of the proenkephalin germ cell promoter using transgenic mice is now under investigation.

Transcription from the upstream promoter of the proenkephalin gene is regulated by the factor AP-1 (*fos/jun*) (45), and expression of both *fos* and *jun* was recently demonstrated in dividing spermatogonial populations (48). It will be of interest to determine whether transcription from both the germ cell-specific and the upstream somatic proenkephalin promoter is also activated in these early spermatogenic cell types.

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